Lactate Dehydrogenase (LDH) Isozymes of Human Atherosclerotic Plaques

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We have been searching for additional markers to explore differences between the smooth muscle cells of human atherosclerotic fibrous plaques and their putative cells of origin in an aortic media and intima. Lactate dehydrogenase (LDH) isozyme analysis was performed on samples of human fibrous plaques selected by gross and microscopic criteria, and significant shifts in M_4/M_2H_2 LDH isozyme ratios were found, relative to the underlying media and adjacent intima specimens. These changes are in the same direction seen in neoplastic tissues *in vitro* and *in vivo* and are probably not secondary to positional factors, inflammatory changes, or degenerative changes. The significance of these findings in relation to the monoclonal hypothesis of atherosclerosis is discussed. (Am J Pathol 1982, 107:316–321)

THE CONCEPT that the proliferation of smooth muscle cells is a primary event in atherosclerotic plaque formation in human beings has emerged in recent years.[1-5] The observation[6] that cells with the morphologic characteristics of smooth muscle cells form the major cellular constituent of human atherosclerotic fibrous plaques[6] was first interpreted as simply a reaction to insudated lipid.[7] Observations on the early stages of development of atherosclerotic plaques of chickens[2] and their resemblance to similar lesions in human coronary arteries[1] provided a reasonable basis for the suggestion that the initial events in the formation of atherosclerosis might be migration and proliferation of smooth muscle cells derived from the media of the artery into the intima. This idea led to our consideration of new mechanisms in the pathogenesis and etiology of at least a substantial proportion of atheromatous plaques. The fundamental questions posed by this shift in viewpoint have changed our focus from the primary importance of lipid insudation to questions concerning factors leading to focal cellular proliferation in artery walls.[4]

In any tissue, one usually considers the following three processes in which an excess number of cells, and therefore proliferation, is a component: 1) reactive proliferation, such as that seen in wound healing or chronic inflammatory processes; 2) hyperplasia, particularly that found in endocrine organs; and 3) neoplastic proliferation. It seems reasonable to consider these possibilities in relation to the pathogenesis

of atherosclerotic plaques. Typical reactive proliferation in the artery wall can be produced by local trauma, such as insertion of a suture[8] or use of a balloon to abrade the intimal surfaces of arteries.[9] Studies of arterial injury produced by balloon catheters have been made, and the evidence has been used in support of the concept that loss of endothelium followed by platelet adhesion to the denuded surface and release of platelet-derived growth factor leads to proliferation of smooth muscle cells of the arterial wall and lesions resembling human atherosclerotic plaques.[3]

The main form of hyperplasia is recognized in endocrine organs, where it can be diffuse or focal. The causes are, presumably, a demand for or stimulation by hormone products. However, the underlying mechanisms are not yet clear. It has been assumed that hyperplasia of smooth muscle cells occurs in arteries. However, it is of interest that with hypertension the number of smooth muscle cells in the arterial wall does not appear to increase, but the number of cells with tetraploid nuclei is substantially increased in both rat and human arteries.[10,11] While hypertension is a "risk" factor in the development of atheroscle-

sis and thus invites investigation, its role in production of focal lesions is not clear.

A significant number of benign neoplasms have been examined and found to have characteristics of clonal cell proliferations. The method involving the use of mosaic individuals is applicable to human tissues. The X-linked polymorphism of the enzyme glucose-6-phosphate dehydrogenase (G-6-PD) provides the tool for exploring the characteristics of cell populations in tissues[12] of certain individuals. The mosaic analysis applied to human atherosclerotic plaques yielded data indicating that in many instances the plaques have a clonal character, whereas the artery wall cell population from which the plaque cell population derives is clearly a finely mixed cell population.[4,5]

The observation that human atherosclerotic fibrous plaques have in many instances a monoclonal character forms a reasonable basis for exploring the hypothesis that these lesions arise by a process related to neoplasia rather than by purely reactive proliferation. This observation suggests that we look for new categories of factors, such as chemical factors or viruses, operating in the production of atherosclerosis.[4,5] The data have been independently confirmed by Pearson et al.[13] and Thomas et al,[14] and the method has been extended to investigations of animal models of atherosclerosis.[15]

The statistical nature of the mosaic analysis and its theoretic bases and limitations we have discussed[5]; its limitations have led us to seek additional ways in which to define possible phenotypic and genotypic differences between smooth muscle cells of plaques and their putative cells of origin in the media and/or intima. Here we report striking differences in lactate dehydrogenase (LDH) isozyme ratios as a distinguishing feature of human fibrous plaques.

Materials and Methods

Human arteries with fibrous plaques were obtained postmortem from the King County Medical Examiner's Office and the Autopsy Service of the University Hospital, University of Washington, Seattle, from white male and female individuals ranging in age from 35 to 55. Postmortem intervals ranged from 4 to 18 hours. Distal abdominal aortic segments were removed and incised longitudinally along the anterior margin, and the intimal surface was gently wiped free of blood and endothelium with a clean tissue. Within 2 hours of tissue retrieval (the specimen being kept on ice during the interim), the intima, together with its fibrous plaques, was stripped en bloc from the underlying media. Full-thickness media samples (0.2 × 0.2 × 0.2 cm) directly beneath or adjacent to plaques
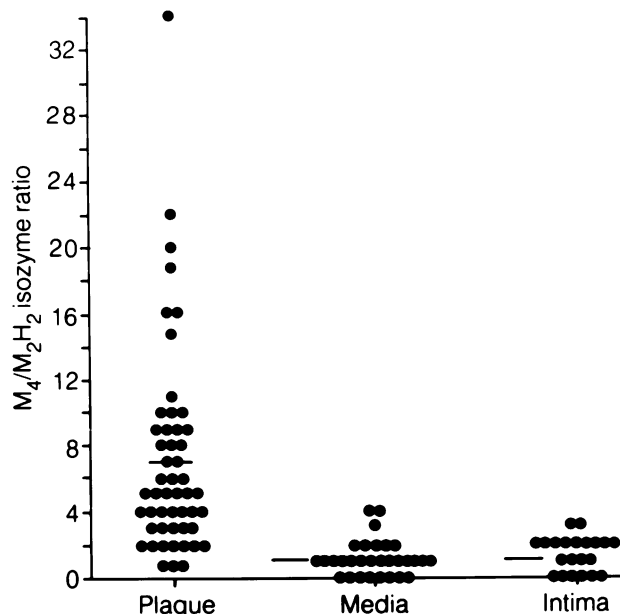

Figure 1—LDH isozyme ratios expressed as M_4/M_2H_2 for plaque, media, and intima samples. Plaques ($n = 48$), 7.20 ± 6.46; media ($n = 30$), 1.09 ± 1.01; intima ($n = 21$), 1.19 ± 0.89 (mean ± standard deviation). Bars indicate the mean for each group.

were obtained initially, with adjacent samples used for microscopy. Fibrous plaques were identified grossly in a fashion analogous to that of Pearson et al.[13] Only those grossly free of ulceration and/or thrombi were sampled. These were generally gray-white, moderately firm, and had a variably sized yellow base. When the yellow base was clearly identifiable, only the gray–white fibrous cap was employed for isozyme analysis, although small lesions (<0.1 cu cm) were used in toto. Specimens were bisected. Half was treated for light-microscopic examination by fixation in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4, followed by methacrylate embedding. In the case of large plaques (>0.5 × 0.5 × 0.2 cm), two or occasionally three samples were used for isozyme analysis. Following homogenization of the second portion of each tissue, LDH isozymes were separated by agarose-gel electrophoresis and visualized by direct staining, as described elsewhere.[16] Isozymes were quantitated by scanning gels in a Beckman RB-2 densitometer followed by digitization of scan outputs in a Hewlett-Packard 9830 desktop computer. Data were expressed as the ratio of bands M_4 to M_2H_2. Analysis of data was by the Student t test.

Results

As shown in Figures 1 and 2, analysis of the M_4/M_2H_2 LDH isozyme ratios in 48 plaques, originating from ten individuals, showed a mean of 7.20, while

M_4 M_3H M_2H_2 MH_3 H_4

a

b

+
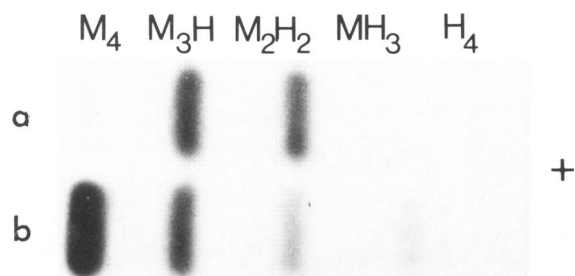
Figure 2 — Representative LDH isozyme electrophoresis pattern following enzymatic staining for media (a) and plaque (b).

the corresponding values for the 30 samples of media and 21 samples of intima were 1.02 and 0.79, respectively. The difference between the plaques and either media or intima was significant at $P < 0.001$, while the difference between media and intima was not significant ($P > 0.05$). These samples were drawn from a larger number; we screened this original population by light-microscopic examination without knowledge of the isozyme pattern. Those samples with poor cellularity or those in which it was determined that inflammatory cells, especially monocytes, constituted more than 20% of the cells present were eliminated

from the study; these represented only a few of the grossly selected plaques (approximately 10%). In this manner, only uncomplicated plaques composed in large part of smooth muscle cells and extracellular fibrous elements were selected (see Figure 3). A subset of these plaques, ranging in size from 0.02 to 0.5 cu cm, was analyzed for the possible relationship of isozyme ratios to plaque size. No significant relationship was found (Figure 4). The relationship of isozyme ratios in plaques to those in the underlying media was also examined, and no significant correlation was found (Figure 5). In a small number of the larger (>0.1 cu cm) plaques, separate superficial and deep plaque samples were used for LDH analysis; no consistent relationship was found (Table 1). Furthermore, no consistent clustering of high or low isozyme ratios in the plaques from a given aorta was noted.

Discussion

We would like to discuss the possible explanations of the observed isozyme shift, with particular reference to the monoclonal hypothesis of atherosclerosis.

It is possible, but unlikely, that cellular infiltration
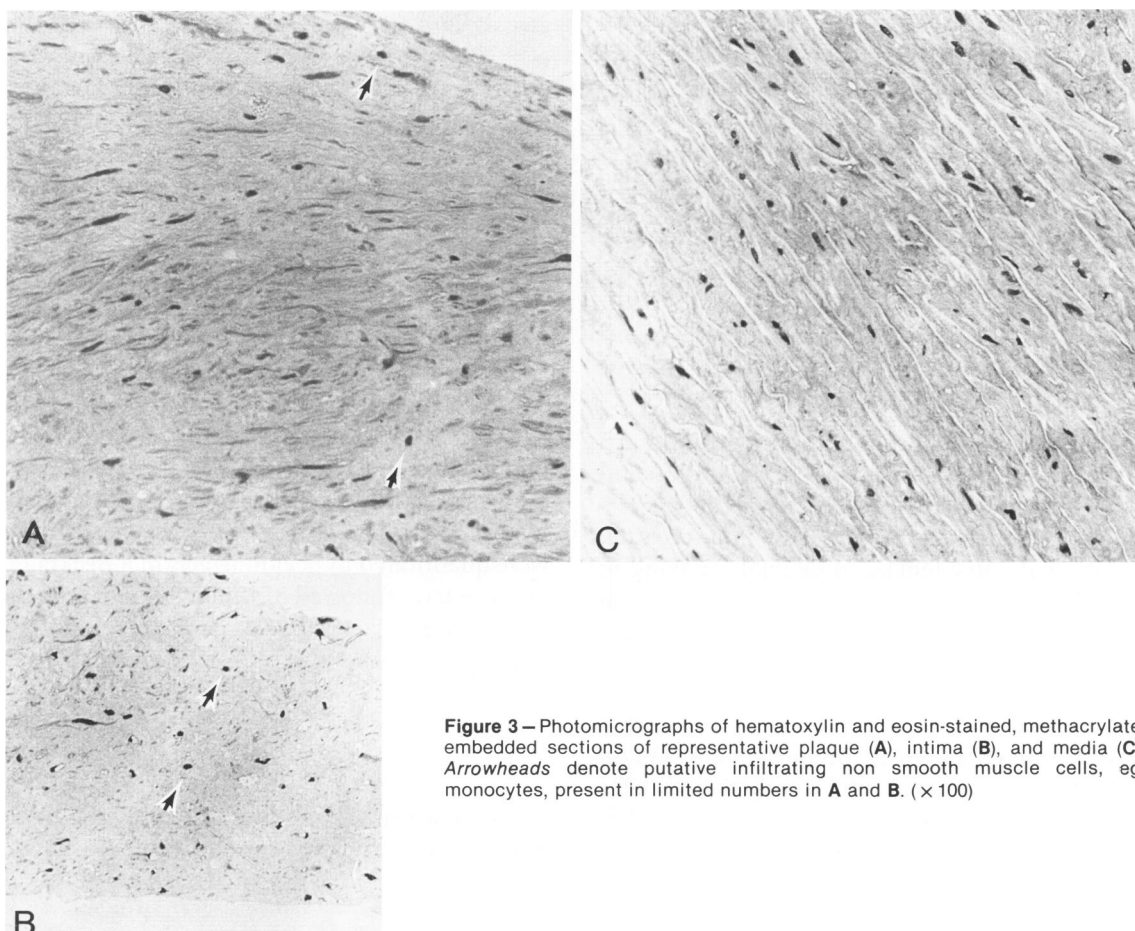
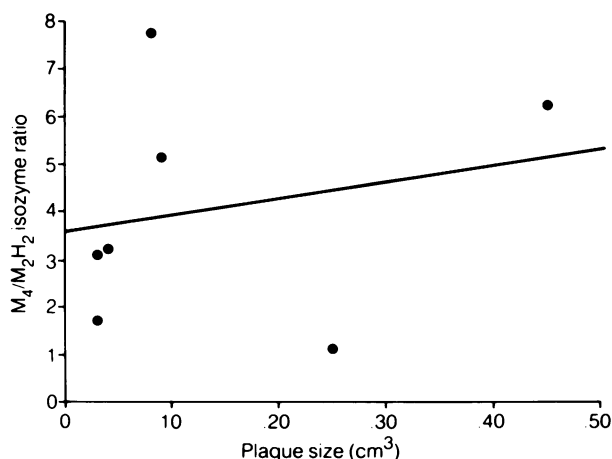Figure 3 — Photomicrographs of hematoxylin and eosin-stained, methacrylate-embedded sections of representative plaque (A), intima (B), and media (C). *Arrowheads* denote putative infiltrating non smooth muscle cells, eg, monocytes, present in limited numbers in A and B. (× 100)
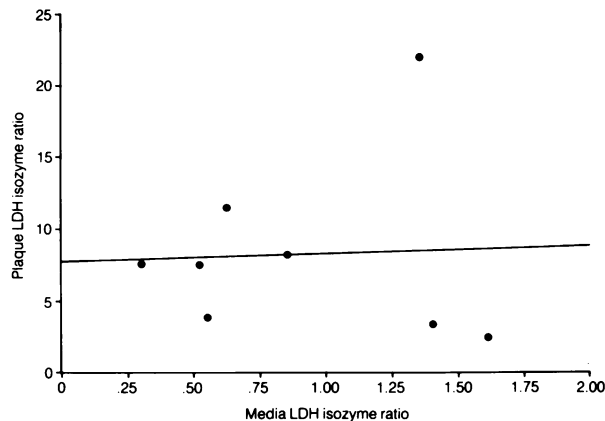
is the source of the M_4-enriched LDH observed in human plaques. Human atherosclerotic plaques are known to contain a fair number of infiltrating, blood-borne cells, eg, monocytes, within the lesions.[17] The effect of the presence of these cells in lesions was minimized by rejection of those cases with substantial numbers of monocytes. Many of the plaques excluded from the study, in which large numbers of inflammatory cells were present, displayed LDH enzyme activities too low to be electrophoretically quantitated, suggesting that smooth muscle cells might display a greater LDH activity per cell than monocytes or lymphocytes. Isolated, defined cell populations will be required for us to definitely address this problem. While, admittedly, histologic study alone is a crude method of quantitating monocytes and lymphocytes, blood monocytes, like lymphocytes,[18] display an LDH isozyme pattern weighted in the direction of M_3H (Gown, unpublished observations), making them indistinguishable from medial or nonatheromatous smooth muscle cells by their LDH isozyme pattern. Furthermore, these cells were present in comparable numbers in the nonatheromatous thickened intima (see Figure 3), where isozyme shifts were not detected.

Although the number of larger plaques examined here is small, the data show the isozyme ratios to be largely independent of lesion size; the LDH isozyme shift thus does not appear to be a secondary phenomenon arising from degenerative changes in the plaques. Indeed, the most striking isozyme shifts were occasionally found in very small, highly cellular plaques with minimal lipid accumulation and monocyte infiltration. Thus, the LDH isozyme shifts reflect a phenotypic change in the smooth muscle cell population that constitutes the plaque.

As recently discussed by Plum and Ringoir,[18] LDH

Figure 5 — Relationship of plaque LDH M_4/M_2H_2 isozyme ratio to that of underlying media. Correlation coefficient = 0.05.

isozymes have stimulated interest because their distribution in a given group of cells may reflect three different and independent types of cellular change: metabolic modulation, altered cellular differentiation, and neoplastic change. These are all of interest in the genesis of the human atherosclerotic fibrous plaque.

Since the M and H subunits are each distinct gene products, the relative amount of each produced is a phenotypic characteristic of a given cell. The relative expression of the M and H genes and hence the relative amounts of M_4 and other isozymes found in a given cell population may in part reflect the microenvironment, especially the local partial pressure of oxygen and hence the relative dependence of the cell on aerobic versus glycolytic metabolism. This phenomenon has been demonstrated, in a reversible fashion, *in vitro* and *in vivo*.[19,20] In the latter, positional factors, ie, the proximity of cells to arterial blood supply, might thus determine LDH isozyme content, as demonstrated recently in the brain.[21]

The arterial wall has been thought to be a tissue exhibiting a negative Pasteur effect and to be highly dependent upon glycolysis. Furthermore, microarchitectural factors are felt to further restrict oxygen delivery. Direct measurement of oxygen tension in rabbit aortic walls by microprobe analysis[22] has revealed that the lowest oxygen tension is in the mid-media,

Figure 4 — Relationship of LDH isozyme ratios to plaque size, representing the subset of plaques with the best cellularity. Correlation coefficient = 0.22.

Table 1 — LDH M_4/M_2H_2 Ratios of Larger Atherosclerotic Fibrous Plaques

Number	Superficial	Deep
1	0.7	0*
2	4.7	22.7
3	5.1	4.5
4	4.9	6.0
5	1.1	5.0
6	1.4	0*

* LDH activity too low to be visualized and quantitated on electrophoretogram.

with a relatively steep gradient across the endothelium. This is consistent with the notion that the oxygen supply of the aortic wall is derived from two sources: the inner third by diffusion from the lumen and the outer two-thirds from the adventitia and vasa vasora.[23] Lodja and Fric[24] examined the relationship between these perfusion relationships and LDH isozymes and observed a disparity between observed LDH isozyme patterns and what they believed were local rates of oxygen delivery based on anatomic considerations, ie, wall thickness and blood supply. Instead, the LDH isozymes seemed to represent a fixed phenotypic marker of the cells of a given species. Thus, even in the normal situation, factors other than metabolic modulation by external control factors must be invoked to explain LDH isozyme patterns.

Diminished oxygen tensions have been reported in experimental aortic lesions made in rabbits by combined mechanical injury and cholesterol feeding,[25] and it has been reported that shifts in arterial LDH isoenzyme patterns can be induced by exposure of animals or of arterial wall smooth muscle cells in culture to lowered oxygen tensions.[20,26] This form of modulation of LDH isozyme patterns does not appear to pertain to the human vascular lesions in our study. First, in the results obtained, the pattern of M_4 preponderance in plaques is not related to the size or depth of the plaque, as might be expected if oxygen diffusion to cells were the conditioning factor. Second, the plaques are closer to the lumen than the underlying media and hence have better access to oxygen diffusing from the lumen; yet the plaques have a substantially higher $M_4:M_2H_2$ ratio than the underlying media. Thus, neither size nor positional features seem to induce a glycolytic shift based upon lowered oxygen tensions governed by tissue mass or positional features.

Though there may be other metabolic modulators of LDH isozyme expression yet to be identified that operate in the artery wall, we would like to consider other possible effectors.

LDH isozyme analyses have been made of cells in various states of differentiation. Thus, independently of the external Po_2 or positional factors, changes in isozyme ratios have been observed in differentiating cell populations such as lymphocytes[18] and erythrocytes.[27] It is in these systems that the variable expression of LDH isozymes has been demonstrated to be a phenotypic marker that appears to be fixed for a given state of differentiation.

Consistent LDH isozyme changes have been reported as a characteristic of neoplastic change in a wide variety of human tissues.[28] The idea that LDH isozyme changes are a function only of positional factors such as local anoxia secondary to abnormal tumor growth and loss of vascularization are not borne out by the presence of these LDH isozyme changes in carcinoma-in-situ lesions of the cervix[29] and in preneoplastic gastric lesions.[11] In an exhaustive review of the data, Schapira[28] showed that there was not a strong correlation between the high glycolytic rate of tumor cells and their LDH isozyme shifts. The pattern instead seemed to represent a "reversion" to fetal patterns.[31] This reversion to the fetal pattern is illustrated in the case of the liver: adult liver cells, unlike most cell populations, display a great excess of the M_4 band. Liver cell carcinomas show isozyme shifts in a direction opposite that of most other malignancies but in the same direction as fetal liver cells.[32] Furthermore, Prasad et al[33] have described LDH isozyme shifts in SV40 and 7,12-dimethylbenz(a)anthracene-induced tumors of mice cultured in vitro, and Caltrider and Lehman[34] have demonstrated increased M_4 LDH isozyme expression in SV40-transformed Chinese hamster cells.

Thus our data clearly demonstrate that a shift in LDH isozyme expression that is qualitatively similar to that described in human neoplasms is present in human aortic fibrous plaques. One can reasonably interpret this as a "marker" of a fixed cellular alteration, distinguishing the plaque smooth muscle cells from the cells resident in their most likely sites of origin, the underlying media or the thickened but nonatheromatous intima.

When coupled with previous data of ultrastructural morphologic modification[2] and single G-6-PD isozyme[4,5,13] within human plaques, a stronger pattern emerges. It is interesting in this regard that approximately two-thirds of the plaques (Figure 1) show LDH isozyme shifts significantly above that of the media and intima, which is roughly the same proportion that were of a single phenotype in the original[4] and later studies using G-6-PD. And just as Pearson et al have described a subset of plaques with an "intermediate" G-6-PD phenotype,[13] no specific LDH isozyme ratio could be used as an absolute "cutoff" between plaque and nonplaque tissue. The reasons are not clear but may relate to heterogeneity in histologically defined fibrous plaques.

Although the same criteria were used in selecting plaques in given individuals in this and the previous G-6-PD studies,[4,5] the latter required the use of black females only. The current study utilized white males and females, precluding simultaneous evaluations of the two markers. The two studies utilize different approaches to the analysis of atherosclerotic plaques but are complementary in their results. Nonetheless, other markers need to be discovered, perhaps ana-

logous to those used in studies of the development of neoplasms in rat livers following carcinogen administration,[35] in which early lesions are identifiable only by their altered enzyme phenotypes (eg, glucose-6-phosphatase or adenosine triphosphatase activity).[36]

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Acknowledgments